Biochimica et Biophysica Acta, 692 (1982) 252–262
Elsevier Biomedical Press

BBA 71366

HIGH-RESOLUTION ^{13}C NUCLEAR MAGNETIC RESONANCE STUDIES OF SMALL UNILAMELLAR VESICLES CONTAINING GLYCOPHORIN A

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(Received April 13th, 1982)

Key words: Lipid-protein interaction; Phosphatidylcholine; Phosphatidylethanolamine; ^{13}C-NMR; Glycophorin; Membrane vesicle

Glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane, has been incorporated in small unilamellar vesicles containing phosphatidylcholine and phosphatidylethanolamine in varying proportions. Hydrocarbon chains of these two lipids have been selectively enriched with ^{13}C and ^{13}C-NMR spin relaxation parameters have been monitored in the presence and absence of protein. Perturbations to ^{13}C line-widths and spin-lattice relaxation times are found to be small and consistent with relatively weak interactions. The perturbations, though small, show some specificity. The carbonyl carbons in both phosphatidylcholine and phosphatidylethanolamine are broadened, but in addition the olefinic carbons in phosphatidylethanolamine are broadened.

Introduction

Interactions between proteins and lipids are obviously important for maintenance of the structural integrity of membranes and for the proper functioning of many membrane proteins. Among the possible ways of characterizing lipid-protein interactions spectroscopic measurements offer an advantage in that they reflect interaction at a very fundamental level and in many cases do so without the need of physical or chemical modification.

Among the spectroscopic methods for probing lipid dynamics, ^{13}C nuclear magnetic resonance (NMR) remains a popular tool. There are sound reasons for this. Most ^{13}C carbons reside in methine, methylene or methyl groups where dipolar interactions with directly bonded protons dominate spin relaxation. These dipolar interactions occur with fixed internuclear distances so that relaxation rates can be simply related to group or molecular reorientation. Several procedures for relating spin relaxation to lipid dynamics are now available (Refs. 1–4, 5 and references therein, 6). General conclusions are that spin-lattice relaxation in ^{13}C-NMR reflects primarily the fast intramolecular motions described as axial diffusions or β-coupled *gauche* isomerization of the hydrocarbon chain [2] and that the line-width, or spin-spin relaxation, can reflect slower processes dependent on intermolecular interactions as well as morphological states of the lipid aggregates. Thus, we might expect either line-widths or spin-lattice relaxation of resonances arising from ^{13}C sites on lipids to respond to the presence of protein [7–10]. ^{13}C, which exists at a natural abundance of 1%, also offers the possibility of selective enrichment of lipids or sites on lipids of particular interest to the researcher. In many cases, the behavior of even minor constituents can be observed above the background from many structurally similar lipid molecules.

Recently we have focused our interest on the

Abbreviations used: NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DSS, 2,2-dimethylsilapentane-5-sulfonic acid.

interaction of glycophorin A with membrane lipids (Ref. 11 and references therein). Glycophorin A is a major glycoprotein in the human erythrocyte membrane. It is believed to be transbilayer with a single hydrophobic α-helix available for interaction with membrane lipids (for review, see Marchesi et al. [12] and references therein). Given the now numerous proposals for transmembrane helices in other membrane proteins, the glycophorin containing membrane seems an ideal system for fundamental research. Glycophorin can be reconstituted with a number of lipids to form bilayer structures of a variety of sizes and states of complexity [13–17]. A procedure involving cholate solubilization followed by rapid removal of cholate on a Sephadex column has, however, proven to lead to particularly high levels of protein incorporation [11]. The protein is incorporated asymmetrically in small unilamellar vesicles identical in size to vesicles prepared by a similar method but without protein. Since morphology of preparations is known to affect spin relaxation directly, the similarity in vesicle sizes obtained by this method facilitates association of changes in lipid spin relaxation rates with the presence of protein.

We have thus chosen to employ the cholate solubilization technique to reconstitute glycophorin with two of the most abundant red blood cell lipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). ^{13}C enrichment of PC or PE has been employed to differentiate contributions from the two lipids to degenerate resonances. Comparison of spin-spin and spin-lattice ^{13}C relaxation times in vesicles with increasing amounts of protein indicates a small immobilization of lipid hydrocarbon chains. Results for reconstituted membranes containing PC and PE:PC mixtures are compared. Results are also compared to those obtained using sonicated preparations and to those obtained previously employing ^{1}H-NMR.

Materials and Methods

Materials

Unlabeled phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were isolated from egg yolks and purified on a silicic acid colum according to the procedure of Singleton et al. [18]. Phosphatidylethanolamine enriched at odd carbons or even carbons with ^{13}C (PE*-odd or PE*-even) was extracted from *Escherichia coli* mutants CY2, which had been fed 90% ^{13}C-enriched [1-^{13}C]- and [2-^{13}C]acetate (Merck Isotopes, Montreal, Canada), respectively, following the procedure of Cronan and Batchelor [19]. The resulting PE*-even is estimated to be approx. 60% ^{13}C-enriched based on intensitites of ^{13}C satellites of the methyl resonance in ^{1}H-NMR spectra. The corresponding ^{13}C-labeled PC*-odd and PC*-even were prepared by converting ^{13}C-labeled phosphatidylethanolamine to phosphatidylcholine with methyl iodide [20]. Enrichments in ^{13}C are at a similar level. In some cases, 90% ^{13}C-enriched methyl iodide (Merck Isotopes) was used resulting in labeling of choline methyl carbons in PC as well. All the lipids employed here were shown to be pure by thin-layer chromatography.

Glycophorin A was isolated and purified following the procedures of Furthmayr et al. [21] and Marchesi and Andrews [22]. Methods are detailed in our previous publication [11]. The buffers used were made from chemicals of reagent grade.

Sample preparation

Glycophorin A was incorporated into vesicles by one of two processes previously described [11]. They are bath sonication and cholate solubilization followed by Sephadex G-100 filtration and dialysis. A typical buffer was 100 mM NaCl/100 mM Tris/10 mM EDTA/0.02% w/v NaN_3 at pH 7.4. In cases of dilute samples, only one-tenth the amounts of Tris and EDTA were used in the buffer. Lipid concentrations in the samples prepared by sonication were usually 2–10% w/v in 5-mm NMR tubes. Lipid concentrations for cholate-prepared samples were typically 0.3% w/v. These more dilute samples were prepared in quantities sufficient for 10-mm NMR tubes.

Sample characterization

Samples were characterized primarily by Sepharose 2B column chromatography. Lipid content was determined by the phosphate assay of Bartlett [23], and protein analysis was performed with either Lowry [24] or sialic acid assay [25,26]. In the latter case, a factor of approx. 4.5 was used to convert sialic acid content to glycophorin content [27].

Density gradient ultracentrifugation was also

employed to investigate sample heterogeneity. Step density gradients were prepared using Metrizamide (Aldrich, Milwaukee, WI) as the medium because of the wide range of densities of interest. Four different densities of 0.989, 1.067, 1.149 and 1.24 g/ml were made up from 0, 19, 40 and 63% w/v Metrizamide solutions at 25°C, respectively. The isopycnic centrifugation was carried out on a Beckman L5 – 50 ultracentrifuge with a SW 40Ti rotor at 35000 rpm for 12 h at 20°C. Fractions of approximately 0.5 ml were then collected from the bottom of the centrifuge tube, dialyzed, and analyzed for protein and phospholipid. The densities were simply determined by pipetting and weighing the aliquots from the fractions at 25°C. Errors of approx. ±0.005 for the measured density are estimated.

NMR measurements

All the ^{13}C-NMR measurements were carried out on a Bruker HX-270 superconducting spectrometer operating at 67.8 MHz in the Fourier transform mode and interfaced to a Nicolet BNC-12 computer. The probe temperature was measured to ±2°C in the presence of broadband ^{1}H decoupling power of about 1 W. Typical ^{1}H-decoupled spectra were recorded with quadrature detection using 8K or 16K data points for a sweep width of 15 kHz. An internal ^{2}H signal from ^{2}HO^{1}H was used for the field lock. The free induction decays were time-averaged over approximately 3000 scans for sonicated samples and 20000 scans for cholate-prepared samples, and then Fourier-transformed. Chemical shifts are reported in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethylsilapentane-5-sulfonic acid (DSS).

Spin-spin relaxation rates (R_2) were simply determined by measuring the line-widths at the half-height, $\Delta\nu_{1/2}$, of the resonances in individual spectra, and using $R_2 = \pi\Delta\nu_{1/2}$. For well-resolved major lipid resonances this method worked adequately and errors are estimated at ±10%. For weak resonances, the errors are estimated at ±20% based on repeated runs.

For spin-lattice relaxation time (T_1) measurements, the progressive saturation technique was employed [28]. This procedure requires a minimum of two spectra and offers a means of T_1 determination for dilute, time-consuming samples. Spectra were accumulated with a $(90° - AT)_n$ pulse sequence and with a $(90° - AT - \tau)_n$ pulse sequence in which the 90° pulse duration was 15 μs and τ was three times the acquisition time, AT. Peak heights were taken as a measure of resonance intensities and a single relaxation time was obtained assuming a single exponential relaxation function. Employing S/N ratios in individual spectra, errors of ±20% are estimated for T_1 values determined by this technique.

Results

Characterization of the samples to be used for NMR studies by Sepharose 2B column chromatography has been described previously [11]. Because homogeneity of protein incorporation is an important consideration in interpretation of some of the data to be presented, samples used here were further characterized using density gradient ultracentrifugation. The solid line in Fig. 1 represents the density gradient formed after centrifugation a Metrizamide step gradient at 100000 × g for 12 h at 20°C. Arrows mark the centers of bands in

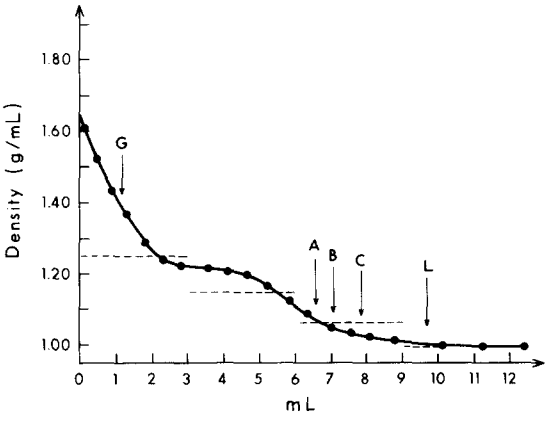

Fig. 1. The density gradient profile formed after ultracentrifugation as described in Materials and Methods. The dashed lines indicate the initial densities of the layers in the centrifuge tube. The arrows denote the bands at equilibrium in individual centrifuge tubes as determined by lipid analysis, protein analysis, or visual inspection. G indicates free glycophorin, L glycophorin A-free vesicles, A cholate-prepared PE : PC with glycophorin A at a lipid: glycophorin A mole ratio of 40 : 1, B and C sonicated PE : PC with glycophorin A at an initial lipid: glycophorin A mole ratio of 65 : 1, and C cholate-prepared PC with glycophorin A at 65 : 1.

which lipid and protein were found. At equilibrium, a pure lipid vesicle band formed visibly at 1.005 ± 0.005 g/ml (L). A pure protein band formed at 1.40 ± 0.02 g/ml (G), in good agreement with the literature value for the glycophorin A aggregate, 1.47 g/ml [29]. Reconstitutes prepared by sonicating PE:PC 1:2 vesicles in the presence of glycophorin A (1:65 with respect to lipid) (bands B and C), by cholate solubilization with PC at a lipid to glycophorin A ratio of 65:1 (C) and by cholate solubilization with PE:PC 1:2 at 40:1 (A) all gave rise to visible bands at intermediate densities as indicated. Protein and phosphate analyses confirmed their presence. At least two recognizable bands were formed for the sonicated sample at densities of 1.045 and 1.025 g/ml, suggesting sample heterogeneity. In comparison, cholate solubilization gave rise to a single visible band and showed all lipid and protein to reside in a single fraction. The cholate prepared PC sample with glycophorin A at 65:1 centered at 1.025 g/ml, whereas the cholate prepared PE:PC sample with glycophorin A at 40:1 centered at 1.075 g/ml. Comparison of densities of 65:1 and 40:1 samples suggests a density change of 0.05 g/ml, corresponding to a 30% change in mole fraction protein. Given an ability to resolve fractions differing in density by 0.01 density unit, we can place limits on the heterogeneity in protein content at $\pm 10\%$. It is significant that the densities of 40:1 and 65:1 samples are less than what one would predict on ideal mixing of lipid and protein components. This may suggest substantial conformational or solvation changes on entering the membrane.

One might expect differences in homogeneity noted in sonicated and cholate solubilized preparations to extend to diffferences in perturbations on ^{13}C-NMR spectra. For this reason, spectra of both types of preparation were examined in detail. Fig. 2 presents a natural abundance ^{13}C-NMR spectrum of 3% sonicated egg yolk phosphatidylcholine vesicles at 30°C. The assignment for these resonances can be readily derived from the literature [30]. Beginning at the low-field end, major resonances are as follows: carbonyl composed of two partially resolved peaks stemming from molecules on the outer and inner halves of the bilayer (approx. 174 ppm); a group of olefinic

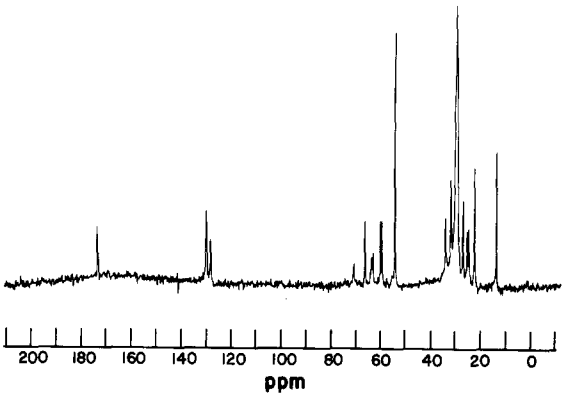

Fig. 2. Broadband-noise ^{1}H-decoupled natural abundance ^{13}C-NMR spectrum of 3% sonicated egg yolk phosphatidylcholine vesicles at 30°C. The spectrum was obtained as described in the Methods section.

carbon resonances from the unsaturated fatty acyl chains (approx. 130 ppm); a group of resonances from glycerol and headgroup carbons (60–70 ppm); Tris (60.1 ppm); choline methyl (54.6 ppm); C_2 methylene (34.5 ppm); penultimate methylene $C_{\omega-2}$ (32.4 ppm); mid-chain methylenes (30.3 ppm); methylene adjacent to one (27.7 ppm) or two olefinic sites (26.0 ppm); C_3 methylene (25.4 ppm); terminal methylene $C_{\omega-1}$, (23.1 ppm); and terminal methyl C_ω (14.3 ppm).

In mixed-lipid vesicles of PC and PE a very similar natural abundance spectrum is observed, as the aliphatic resonances (15–30 ppm) from both molecules are nearly superimposable. It is possible, however, to both improve sensitivity and selectively observe resonances from one molecule by biosynthetic enrichment. Fig. 3a shows the 25°C spectrum of a mixed PE:PC 1:2 (approx. 1%) sonicated vesicle preparation having the PE labeled at odd carbons. The apparent absence of C_2 and $C_{\omega-2}$ and a much diminished terminal methyl resonance supports interpretation as a spectrum arising predominantly from the PE component. The apparent simplification of the olefinic region results from the absence of large amounts of polyunsaturates in *E. coli*-derived lipid. Similar selectivity can be obtained if only even carbons are enriched. The PC component can also be selectively observed by using PE with natural abundance carbon and PC with either odd or even carbons enriched.

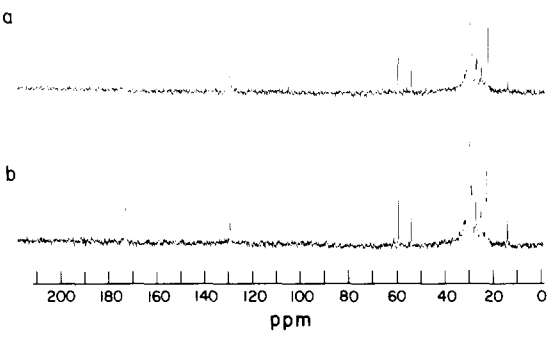

Fig. 3. The ^{1}H-decoupled ^{13}C NMR spectra of approx. 1% sonicated PE*-odd: PC 1:2 vesicles at 25°C, (a) in the absence of glycophorin, (b) in the presence of glycophorin incorporated as described in the Materials and Methods section at a lipid: glycophorin A mole ratio of 65:1.

Simple addition of glycophorin to vesicle preparations such as those discussed above produces no noticeable changes in spectra. Elution of such a sample on a Sepharose 2B column results in separation of lipid and protein components, indicating only weak association. A more vigorous treatment obviously must be employed to effect glycophorin incorporation. Extensive sonication is the most straightforward means of effecting incorporation. After an extra hour of sonication in a bath sonicator below 40°C, a preparation results in which protein and lipid components cannot be separated by column chromatography. Despite the fact that isopycnic centrifugation shows some heterogeneity, this sample warrants investigation.

Fig. 3b presents a spectrum of a sample analogous to that in Fig. 3a except that glycophorin has been incorporated to a lipid-to-protein mole ratio of 65:1 by sonication. Changes in the spectrum are small but easily distinguisable on expansion of selected regions. The main changes in Fig. 3 are a measurable broadening of the C_3 resonance (25.4 ppm), from a line-width of 42 to 50 Hz, and a broadening of the carbonyl resonance, from 35 to 45 Hz, which results in loss of resolution of components from inner and outer halves of the bilayer. A similar effect has been found for the C_2 carbon (20% broadening at a lipid: glycophorin A ratio of 100:1) when PC is labeled at even positions on the hydrocarbon chain in the PE:PC 1:2 system. No noticeable broadening of resonances from carbons further down the hydrocarbon chain is observed. Effects of sample heterogeneity are not obviously present as multiple component resonances, but magnitudes of broadening did vary from preparation to preparation. In all cases, largest effects were noted from carbons near the interface.

Similar spectra were obtained for samples prepared by cholate solubilization. For a PC-odd enriched sample at 37°C with and without protein at a lipid-to-protein ratio of 55:1, differences in spectra were very small. There was no noticeable selective broadening of the C_3 resonance in the aliphatic region, nor was any intensity loss noted. On expansion broadening of the carbonyl resonance was again apparent, but was consistently smaller than that observed for comparable sonicated samples. More significant was the fact that on expansion of the olefinic carbon resonance (approx. 130 ppm), a degree of broadening not observed in sonicated samples was apparent. The extent of broadening can be enhanced both by lowering temperature and by increasing the amount of protein. As shown in Fig. 4, at 40:1 lipid-to-protein ratio and 25°C, the olefinic peak from odd-carbon-labeled PC in a pure PC vesicle is broadened by 80% (from 53 to 96 Hz). At 12°C and 60:1 lipid-to-protein ratio, there is 50% broadening for the olefinic resonance, and slight (at most 20%) protein-induced broadening of resonances arising from aliphatic carbons becomes apparent.

It is important to note that magnitudes of perturbations in the data on cholate prepared vesicles are quantitatively more reproducible than perturbations in sonicated samples. Perturbations are also qualitatively different in that resonances arising from carbons near the surface of the bilayer are not perturbed as much and resonances arising from the olefinic carbons, which lie closer to the center of the bilayer, are perturbed to a greater extent. Given the improved reproducibility, we focus our remaining experiments on cholate-prepared vesicles.

In particular, we would like to contrast the behavior of PE and PC in mixed lipid vesicles prepared by the cholate solubilization method. Fig. 5 shows an expansion of carbonyl and olefinic regions for 1:2 PE:PC samples, with no glycophorin and with glycophorin at an 80:1 mole ratio. A moderately low temperature (25°C) is

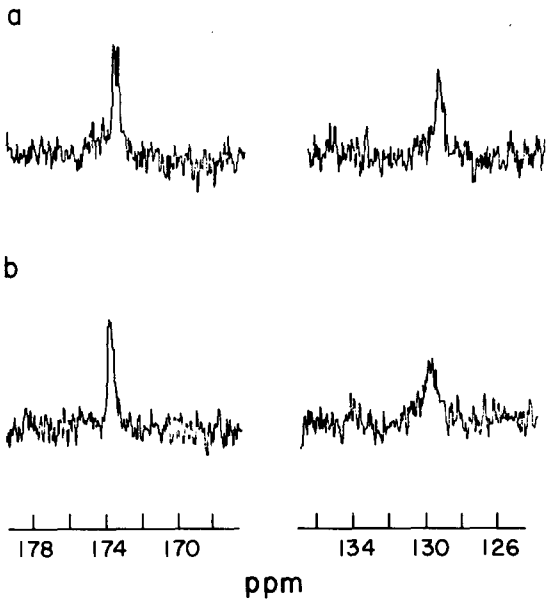

Fig. 4. Expanded low field region of the ^{1}H-decoupled ^{13}C-NMR spectra for the PC*-odd vesicles prepared by cholate solubilization at 25°C (a) in the absence of glycophorin, (b) in the presence of glycophorin incorporated at a lipid: glycophorin A mole ratio of 40:1.

used to enhance perturbations, and the PC has been enriched at odd carbons. Some broadening of the carbonyl is apparent, but broadening of the olefinic resonance is very small in comparison to that shown in Fig. 4. No effects on the aliphatic region are apparent. This can be compared to the preparation containing only PC shown in Fig. 4, where 80% broadening of the olefinic region is observed at 40:1 lipid-to-protein ratio. Protein content in the present case is a factor of two lower. Nevertheless, models of protein-lipid interaction based on rapid exchange of boundary and bulk lipid would have predicted 40% broadening, which we clearly do not see. The results might suggest a more favorable interaction of glycophorin with the PE component.

Fig. 6 shows carbonyl and olefinic regions for a PE:PC 1:2 sample in which the PE has been enriched a odd carbons, and glycophorin has been incorporated by the cholate method to a level of 80:1. Broadening of the carbonyl is again apparent, but now approx. 50% broadening of the olefinic resonance also occurs. Although some

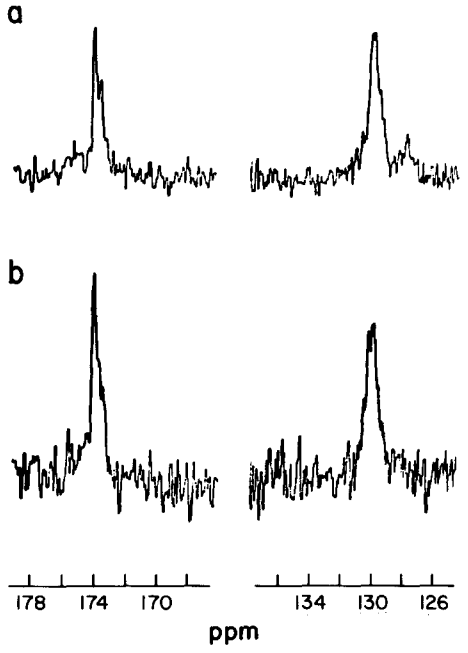

Fig. 5. Expanded low field region of the ^{1}H-decoupled ^{13}C-NMR spectra for the PE:PC*-odd 1:2 vesicles prepared by cholate solubilization at 25°C (a) in the absence of glycophorin, (b) in the presence of glycophorin incorporated at a lipid: glycophorin A mole ratio of 80:1.

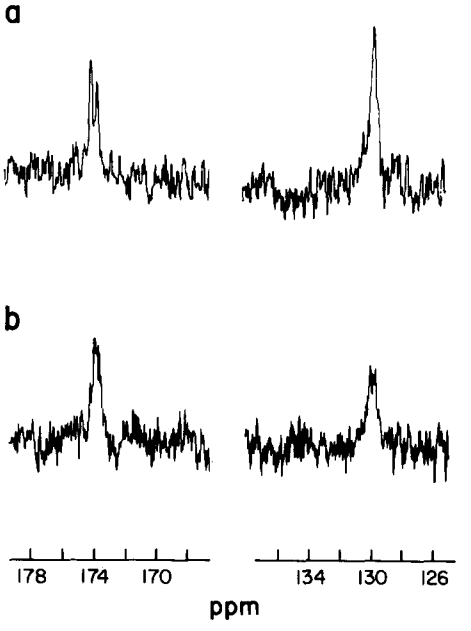

Fig. 6. Expanded low field region of the ^{1}H-decoupled PE*-odd: PC 1:2 vesicles prepared by cholate solubilization at 25°C (a) in the absence of glycophorin, (b) in the presence of glycophorin incorporated at a lipid: glycophorin A mole ratio of 80:1.

variation in line shape near the base line in the aliphatic region is present, no measurable increase in line-width at half-height is observed. Comparison with Fig. 4 suggests a similar type of interaction for PC and PE, but in mixed vesicles there appears to be some preference for interaction of PE with glycophorin over PC with glycophorin.

In order to elucidate further the nature of the interaction between lipid hydrocarbon chain and membrane incorporated glycophorin A, spin-lattice relaxation times were measured for carbon sites in samples analogous to those discussed above, using the progressive saturation technique [28]. Results are presented in Table I along with error estimates based on signal-to-noise measurements in individual spectra. Magnitudes and trends in the data for PC vesicles at 25°C are consistent with data published by other authors on PC vesicles [30,31]. Results for the PE component of mixed PE::PC vesicles at 25°C are in agreement with PC results except for possibly the $C_{\omega-1}$, which has a shorter relaxation time for PE.

Lowering temperature in the PE:PC sample from 25 to 12°C produces a significant shortening of T_1, indicating that motions dominating T_1 relaxation are on a time scale short compared to $1/\omega_0 \approx 10^{-9}$ s.

Preparations of PE:PC vesicles at 25°C having increasing amounts of protein show a trend toward shorter T_1 values for most methylene carbons. Magnitudes of changes are, however, small. At 40:1 lipid-to-protein ratios, a factor of 2 more protein than in samples which show 50% broadening of the olefinic PE resonance, only the $C_{\omega-1}$ shows a significant shortening of the T_1 ($35 \pm 15\%$). At a ratio of 20:1, a factor of 4 more protein, the T_1 of the olefinic carbon decreases by $50 \pm 50\%$, as do T_1 values of other carbons in the hydrophobic region. Thus, for comparable protein ratios, T_1 changes are smaller than changes in line-widths. At very high protein levels, T_1 values are reduced, but the reduction is significant throughout the hydrocarbon region and not localized to the olefinic carbons.

Discussion

The above data demonstrate that ^{13}C-NMR can be used to monitor lipid-protein interactions in vesicle systems prepared under a variety of condi-

TABLE I

^{13}C-NMR SPIN-LATTICE RELAXATION TIME MEASUREMENTS FOR CHOLATE-PREPARED SAMPLES

Values (for carbon) are in s. GPA, glycophorin A.

Sample	$N(CH_3)_3$	C_2	C_3	C_n	C=C	C=C-C	$C_{\omega-2}$	$C_{\omega-1}$	C_ω
PE*:PC [a,b]	0.35	0.09	0.20	0.30	0.38	0.30	0.31	0.70	1.21
12°C	±0.08	±0.05	±0.03	±0.04	±0.08	±0.03	±0.04	±0.11	±0.24
PE*:PC/GPA	0.40	0.22	0.24	0.27	0.50	0.38	0.28	1.07	1.21
80:1, 12°C	±0.08	±0.05	±0.05	±0.02	±0.13	±0.06	±0.03	±0.20	±0.24
PE*-odd:PC	0.27		0.38	0.43	0.54	0.54		0.94	
25°C	±0.05		±0.03	±0.03	±0.13	±0.06		±0.13	
PE*-odd:PC/GPA	0.54		0.27	0.38	0.57	0.46		0.60	
40:1, 25°C	±0.16		±0.04	±0.06	±0.14	±0.06		±0.07	
PE*-odd:PC/GPA	0.30		0.19	0.25	0.27	0.16		0.30	
20:1, 25°C	±0.14		±0.10	±0.05	±0.13	±0.05		±0.08	
PC*-odd	0.40		0.43	0.33	0.47	0.60		1.90	
25°C	±0.05		±0.10	±0.03	±0.14	±0.16		±0.81	
PC*-odd/GPA	0.37		0.31	0.35	0.32	0.43		1.35	
40:1, 25°C	±0.03		±0.03	±0.03	±0.13	±0.08		±0.40	

[a] PE:PC mixed vesicles with the PE:PC molar ratio 1:2.

[b] T_1 values for the PE*:PC samples were obtained from two seprately run samples, i.e., PE*-odd:PC and PE*-even:PC.

tions. There have been some previous reports using ^{13}C labels to investigate glycophorin A reconstituted systems. These have employed primarily ^{13}C labels on the head group of the lipid. No significant spectral perturbations were reported for glycophorin A vesicles reconstituted by sonication with head-group labels [13]. Brulet and McConnell [32], however, found evidence for mobilization of the head group when glycophorin was incorporated into more extended bilayers by dialysis of cationic detergents [17]. Gerritsen et al. [33] observed no significant spectral effect on the head-group resonance when glycophorin A was incorporated in vesicles by the method of MacDonald and MacDonald [14]. Only recently has a ^{13}C label on the acyl chain been used in glycophorin A reconstitution. Results of Utsumi et al. [10] using the rehydration procedure of MacDonald and MacDonald suggest small immobilization of the hydrocarbon chain and interpret a broad component in ^{13}C-NMR spectra as 'boundary lipid' in slow exchange with bulk lipid. The stoichiometry of lipid-protein interactions in reconstituted liposomes based on this broad component has been estimated to be about 30 lipid molecules per glycophorin molecule, a result consistent with the stoichiometry found in our ^{1}H-NMR studies [11]. We do not, however, find evidence for a slowly exchanging 'boundary lipid'. There are several possible reasons for this discrepancy. First, the sample preparations are different and it is possible that the nature of the lipid-protein complex differs. Second, sample heterogeneities can lead to the appearance of two component resonances. And third, it is possible to obtain non-Lorentzian lines from large vesicle preparations whose broad wings can be mistakenly interpreted as a second component.

Differences between sonicated and cholate solubilized preparations used in the present study emphasize that it is not judicious to assume that protein incorporation is always complete, or always of the same nature, without some independent means of characterization. Isopycnic centrifugation and gel permeation elution profiles show the cholate-prepared samples used here to be homogeneous. Elution profiles are also used to support the homogeneity of the preparations used by Utsumi et al., but microscopic heterogeneities are difficult to rule out. Whether the larger vesicles used by Utsumi et al. should have single Lorentzian lines is open to question. Proton spectra of more extended bilayers have been shown to produce superLorentzian lines, whose broad wings could be interpreted as a second component [34].

We should also point out that if two motionally distinct populations were to contribute independently to a two-component line with a width less than 100 Hz, their lifetimes in the distinct states would have to be longer than 10 ms. We feel that such a long lifetimes is unlikely for a simple protein-lipid association and it is inconsistent with most observations on protein-lipid systems to date [35].

In all studies to date, including those of Utsumi et al. [10], perturbations to lipid line-widths and spin-lattice relaxation times have been quite small, given the high effective ratios of protein to lipid employed. Perturbations to line-widths in cholate prepared vesicles studied here are also small, however, they reach measurable levels (approx. 50% broadening) for olefinic carbons and carbonyl carbons when protein content is greater than 1:60 with respect to lipid on a mole ratio basis. This perturbation, regardless of its origin, can be used to compare interactions with different lipids. Selective enrichment of odd acyl chain carbons first in the PE component of a 1:2 PE:PC vesicle and then in the PC component, has allowed us to compare interactions of glycophorin A with these lipids. Broadening of the carbonyl is similar in each case but the extent of olefinic carbon broadening is greater for PE, suggesting some preferential interaction.

Preferential interaction of a protein with one lipid over another is neither new nor unexpected. Several reports of preferential association of glycophorin with anionic lipids (phosphatidylserine or phosphatidylinositol) exist [36–39]. Jokinen and Gahmberg [40] have found that external labeling of PE in human En(a −)erythrocyte membranes, which lack glycophorin A, is more efficient than in normal membranes. Although a number of explanations exist, this could result from a preferential interaction of PE with glycophorin A. It has been suggested by Gerritsen et al. [33] and Van der Steen et al. [41] that PE in the glycophorin A-lipid reconstitution mixture is necessary to maintain the

ion permeability barrier of resulting vesicles. For other transmembrane proteins, preferential interactions with PE have often been suggested. This is the case in sarcoplasmic reticulum Ca^{2+}-ATPase [9] and in membrane-bound glucosyl and galactosyl transferases of *Salmonella typhimurium* [42,43]. Preferential interaction of glycophorin with unsaturated lipids has also been reported [32]. However, preferential interaction at the unsaturated carbons of PE has not been noted.

Given the small magnitude of broadening and given the absence of experiments which could separate homogeneous and heterogeneous broadening mechanisms, we choose not to comment in detail on the origin of preferential broadening of PE over PC. We will point out that, in addition to motional restriction, heterogeneity in chemical shift is a possibility. Both the carbonyl and the olefinic carbons provide sites with enhanced environmental sensitivity of chemical shift. Heterogeneity due to protein distribution among vesicles has been effectively ruled out, but there may be some heterogeneity in the mode of protein incorporation, in the nature of the fatty acid in which the double bond is located, and certainly in the occurence of lipid on both the inner and outer halves of the bilayer. Since PE preferentially distributes to the inner half of the bilayer, giving nearly equal resonance intensity contributions from each, differences in shifts for inner and outer layers may also partly explain the enhanced broadening of PE resonances over PC resonances. Clarification of these possibilities will have to await further experimentation.

Regardless of the source of broadening, the measured line-width can be taken as a lower limit for homogeneous contributions to T_2. That is, if the protein causes an increase in line-width through restriction of motions, this contribution cannot be any greater than the measured line-width. For the olefinic region, 80% broadening for a PC to glycophorin A ratio of 40:1 at 25°C is comparable to our earlier observation by ^{1}H-NMR that shows approx. 60% broadening for lipid methylene proton resonances under similar conditions [11]. The olefinic value is, however, unique. For the aliphatic region, which should be more directly comparable to proton results, broadening is smaller (no more than 10% for a lipid to protein 40:1 sample). Effects on spin-lattice relaxation times are even smaller; they are not observable at 40:1 mole ratio, but do show a general reduction throughout the hydrophobic region at lipid glycophorin A 20:1 mole ratio.

Given that perturbations to ^{13}C spin relaxation parameters, particularly T_2, are smaller than for protons, any model constructed to explain spin relaxation must account for these differences. One pre-eminent characteristic of ^{13}C spin relaxation is that sites are well shielded from intermolecular interactions and relaxation can usually be treated as resulting from rotational modulation of dipolar interactions with directly bonded protons. For proton NMR, intermolecular interactions must be considered. We suggest that intermolecular interactions can account for the differences in ^{13}C and ^{1}H spin-spin relaxation perturbations. To establish whether or not such interactions are sufficiently large to account for our observations, we consider the following crude model. The membrane-spanning portion of glycophorin will be treated as a cylinder immobile in the membrane on the time scale of vesicle rotation with a surface of proton-bearing nonpolar amino acid residues. The methylene groups of a lipid hydrocarbon chain move rapidly compared to $1/\omega_0$. We will assume we can represent this simply as a rotation which moves the protons over the surface of a sphere of approx. 2 Å radius. The closest distance of approach between protons on the glycophorin A surface and the proton of a methylene group is twice the Van der Waals radius of a proton, 2.4 Å. The farthest distance will correspond to that at the extreme of the sphere representing the lipid methylene. Since lipid rotation is fast, these motions will not contribute directly to line-widths but will simply reduce the magnitude of the protein-lipid intermolecular dipolar interactions. The residual dipolar interactions will be modulated by vesicle tumbling to produce observed line broadening. To calculate intermolecular contributions to widths, we will also assume that averaging predominately affects the apparent internuclear distance. The mean square interaction for a methylene proton with the closest protein proton will then be $\overline{H_D^2} = 3/4\gamma_H^4\hbar^2(\overline{r_{HH}^{-3}})^2$. For like spins, the expression for T_2^{-1} can be approximated as $\overline{H_D^2}\ 3/5\ \tau_c$ [44] where

τ_c, the vesicle tumbling time, is obtained using the Stokes-Einstein equation (approx. $3.3 \cdot 10^{-6}$ s) [45]. This procedure indicates an additional line broadening from one proton on glycophorin A of as much as 60 Hz, a value large enough to explain proton line-width observations. The analogous argument for a ^{13}C nucleus must be modified to account for the fact that the ^{13}C nucleus is removed from the surface of the representative methylene sphere by approximately another C-H bond length (1.07 Å). Also, for unlike spins the approximate expression for T_2^{-1} is given by H_D^2 4/15 τ_c, in which $H_D^2 = 3/4\ \gamma_H^2 \gamma_C^2 \hbar^2\ (r_{CH}^{-3})^2$. Evaluation leads to an estimate of an intermolecular contribution to ^{13}C line broadening of about 1 Hz. It is, therefore, possible to interpret the unequal effects of protein on spin-spin relaxations of ^1H and ^{13}C nuclei on the basis of intermolecular contributions to relaxation.

Thus, line-widths and spin-lattice relaxation times for most carbons in the hydrocarbon region of the lipid bilayer show minimal protein induced perturbations of lipid hydrocarbon chain motion. Since line-width changes observed in proton spectra can have appreciable intermolecular contributions, perturbations shown in ^1H and ^{13}C studies are not necessarily in conflict. The degree of motional restriction on protein-lipid interaction may, however, be even less than previously suggested based on proton data alone. It is noteworthy that the minimal effect of protein on T_1, the increase in line-width due to homogeneous or inhomogeneous broadening mechanisms and the absence of observable boundary lipid in slow exchange with lipids in a liquid crystalline phase observed here are consistent with recent ^2H-NMR studies of rhodopsin and cytochrome *c* oxidase [46,47]. There are apparent inconsistencies with ^{13}C and ^{31}P work on glycophorin [10,48].

Acknowledgements

The authors would like to thank Drs. Heinz Furthmayr and Thomas Schulte for assistance in the purification of glycophorin A. We would also like to thank Dr. V.T. Marchesi for many useful discussions throughout the course of this project. This work was supported by a grant from the Department of Health and Human Services, GM 19035, and by NMR facilities made available through grant CHE 7916210 from the National Science Foundation.

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